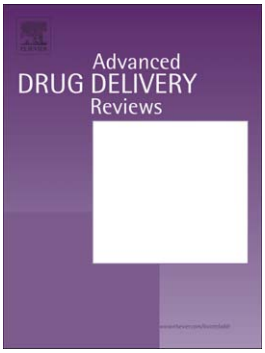

Nano-technology based carriers for nitrogen-containing bisphosphonates delivery as sensitisers of $\gamma\delta$ T cells for anticancer immunotherapy

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Nano-technology based carriers for nitrogen-containing bisphosphonates delivery as sensitisers of γδ T cells for anticancer immunotherapy

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Abstract

Nitrogen containing bisphosphonates (N-BPs) including zoledronate (ZOL) and alendronate (ALD) inhibit farnesyl diphosphate synthase, and have been shown to have a cytotoxic affect against cancer cells as a monotherapy and to also sensitise tumour cells to destruction by γδ T cells. γδ T cells are a subset of human T lymphocytes and have a diverse range of roles in the immune system including the recognition and destruction of cancer cells. This property of γδ T cells can be harnessed for use in cancer immunotherapy through *in vivo* expansion or the adoptive transfer of *ex vivo* activated γδ T cells. The use of N-BPs with γδ T cells has been shown to have a synergistic effect in *in vitro,* animal and clinical studies.

N-BPs have limited *in vivo* activity due to rapid clearance from the circulation. By encapsulating N-BPs in liposomes (L) it is possible to increase the levels of N-BPs at non-osseous tumour sites. L-ZOL and L-ALD have been shown to have different toxicological profiles than free ZOL or ALD. Both L-ALD and L-ZOL led to increased spleen weight, leucocytosis, neutrophilia and lymphocytopenia in mice after intravenous injection. L-ALD was shown to be better tolerated than L-ZOL in murine studies. Biodistribution studies have been performed in order to better understand the interaction of N-BPs and γδ T cells *in vivo*. Additionally, *in vivo* therapy studies have shown that mice treated with both L-ALD and γδ T cells had a significant reduction in tumour growth compared to mice treated with L-ALD or γδ T cells alone. The use of ligand-targeted liposomes may further increase the efficacy of this combinatory immunotherapy. Liposomes targeting the αvβ6 integrin receptor using the peptide A20FMDV2 had a greater ability than untargeted liposomes in sensitising cancer cells to destruction by γδ T cells in αvβ6 positive cancer cell lines.

1. Nitrogen-containing bisphosphonates

1.1 Mechanism of Action of N-BPs

While nitrogen-containing bisphosphonates (N-BPs) were originally used in the treatment of osteoporosis and other bone disorders [1], they have shown to have several clinical indications including cytotoxic activity as a monotherapy [2, 3] and activation of γδ T cells [4]. Bisphosphonates (BPs) are chemically stable analogue of pyrophosphate compounds found in nature. All BPs in clinical use consist of a core structure made up of P-C-P bonds which is resistant to enzymatic hydrolysis (**Figure 1**) [5].

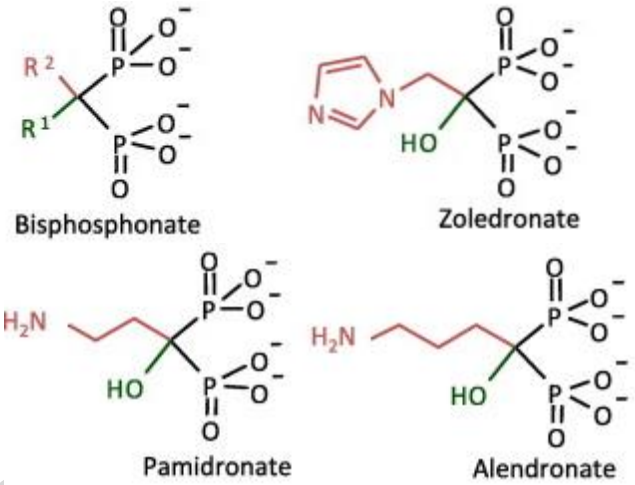

Figure 1: Structure of various bisphosphonates. Template structure of bisphosphonates and structure of zoledronate and other nitrogen-containing bisphosphonates discussed (Adapted from Stresing *et al*, 2007 [6]).

Later generations of BPs led to in incorporation of nitrogen atoms into their structure, increasing their potency due to an additional mechanism of action [1]. N-BPs inhibits farnesyl diphosphate (FPP) synthase which is a key enzyme in the mevalonate biosynthetic pathway [7]. Inhibition of FPP synthase leads to the upstream accumulation of the triphosphoric acid 1-adenosin-5-yl ester 3-(3-methylbut-3-enyl) ester (Appp1) which then induces apoptosis *via* the inhibition of mitochondrial ADP/ATP translocase [8]. Inhibition of FPP synthase also prevents the prenylation of Guanosine triphosphate (GTP)-binding proteins

such as Ras, Rho and Rac leading to cell growth inhibition and can activate the caspase dependant mechanism of apoptosis [9].

1.2 Anti-cancer activity of N-BPs

BPs have also found clinical applications in the treatment of cancer. Both BPs and N-BPs are use in the treatment of multiple myeloma [10] and bone metastases from breast, lung, prostate and other solid tumour cancers [11]. They have shown to be effective in reducing skeletal-related events associated with malignancies such as hypercalcaemia and increased bone destruction [12]. N-BPs have also shown to have anti-tumour activity in non-osseous tumours and have been shown to induce tumour apoptosis and inhibit tumour cell proliferation, migration and invasion. They also have anti-angiogenic effects and interfere with endothelial cell migration, proliferation and tube formation [6]. The use of ZOL and ALD as anti-cancer agents will be focused on in this review.

ZOL is the most potent of the clinically used BPs. due to the presence of two nitrogen atoms contained within a heterocyclic ring structure [13]. ZOL has direct effects on tumour cells *in vitro* and has been shown to decrease the viability and proliferation of several cancer cell lines as well as inducing apoptosis of cancer cells. After exposure of the prostate cancer cell lines PC-3, DU-145, LNCaP and CRW22Rv1 to 100μM of ZOL, the autophagic and apoptotic proteins LC3-II and activated caspase-3 were detected showing that ZOL exposure resulted in cell death [14]. ZOL has also been shown to have a synergistic effect *in vitro* when used in combination with the cytotoxic agents; paclitaxel, etoposide and cisplatin [15].

ZOL has been shown to inhibit progression of established bone metastases and development of new bone metastases in two models of breast cancer in mice [16]. Nude mice bearing

MDA-MB-231 tumours were subcutaneously injected with 0.2, 1.0 or 5.0 μg/day of ZOL for 10 consecutive days and were shown to have reductions in bone lesions of more than 80% compared to controls [16]. Treatment with 5 μg/day ZOL for seven days after injection of 4T1 murine mammary tumour cells were shown to decrease the formation of new bone metastases [16]. Another study in a murine model of multiple myeloma has shown a reduction in osteolysis, tumour burden and angiogenesis with a subsequent increase in survival time [17]. ZOL has also shown to be effective in the treatment of soft tissue tumours in extra-osseous sites. It has been shown to reduce the growth of cervical tumours and the progression of premalignant lesions in a transgenic mouse model [18]. ZOL was shown to inhibit expression of the pro-angiogenic protease, matrix metalloproteinase 9 (MMP-9) by tumour associated macrophages.

Clinically, ZOL has been used in several studies to determine its anti-cancer effects in humans. A phase II clinical study has shown that ZOL increases the clearance and reduces the number of disseminated tumour cells in the bone of patients with early breast cancer [2, 3]. However, other studies have not shown the benefit of adjuvant ZOL treatment in early breast cancer [19]. A clinical trial of the use of ZOL in multiple myeloma, demonstrated 16 % reduced mortality and an extended mean survival of 5.5 months when compared to the non-nitrogen containing BP, clodronate [20]. A Cochrane meta-analysis of other clinical trials confirmed the therapeutic efficacy of ZOL in myeloma patients [21]. ZOL has also shown to be effective in the treatment of malignancy-related skeletal issues in acute myeloid leukaemia [22] and lymphoma [23]. Additionally, prostate cancer patients treated with ZOL were shown to improved prostate-specific antigen progression-free survival time [24]. Various trials in patients with bone metastasis from solid malignancies have shown the benefit of ZOL therapy [25].

ALD has also been shown to have anti-cancer activity in both *in vitro* and *in vivo* studies. When human umbilical vein endothelial cells (HUVEC) were treated with ALD, migration and formation of capillary-like structures were inhibited *in vitro* [26]. This is thought to be due to the inhibition of Rho geranylgeranylation. *In vitro* inhibition of migration of the prostate cancer cell lines PC-3 and Du-145 and the breast cancer cell lines MDA-MB-231 after treatment with ALD has also been observed [27]. ALD inhibited the proliferation of the Huh-7 hepatocellular carcinoma cell lines in a time and dose dependant manner at concentrations between 5-20 μM [28]. ALD has also been shown to have anti-proliferative effect on the epidermoid carcinoma cell line A431, as well as inhibiting cell invasion in a matrigel invasion assay [29]. ALD downregulated MMP-2 in osteosarcoma cell lines, leading to inhibition of cell invasion [30]. ALD has been shown to have synergistic cytotoxic effect with simvastatin on PC-3 cells when used at low doses due to sequential blockade of the mevalonate pathway [31].

ALD (0.5 mg/kg, s.c.) was shown to reduce tumour growth and decrease tumour metastasis to prostate-draining lymph nodes in an orthotopic PC3 nude mouse tumour model [32]. A decrease in the number of CD-34 positive endothelial cells and an increase in apoptotic cells in the tumour and lymph nodes was observed, suggesting both cytotoxic and anti-angiogenic effects. When nude mice inoculated with i.p. Caov-3 ovarian cancer cells were treated i.p. with ALD, an anti-tumour effect was observed [33]. Stromal invasion of the tumour was decreased and MMP-2 activity was inhibited in the ascites. In the same model, ALD treatment has also shown to reduce intra-tumour neo-angiogenesis in disseminated ovarian tumours of athymic mice [26]. In clinical studies, ALD has been shown to increase bone mineral density of the spine and hip in men with prostate cancer treated with androgen

deprivation therapy [34, 35]. However, the direct anti-tumour efficacy of ALD has not yet been studied in humans.

1.3 Pharmacokinetics of BPs

The anti-tumour efficacy of N-BPs for non-osseous tumours is limited by their pharmacokinetic properties. For example, although ZOL has been shown to have anti-tumour activity *in vitro*, its limited *in vivo* efficacy is probably due to its rapid renal clearance and accumulation in the bone [36]. When given orally, less than 1 % of N-BPs are absorbed [37] and this is decreased in the presence of food and drinks containing calcium, magnesium or aluminium [38]. BPs can be administered intravenously to circumvent their poor absorption. However, BPs that are not excreted renally bind rapidly to the bone [39]. N-BPs are not metabolised and are excreted unchanged mainly in the urine, with a small percentage excreted in the bile [39]. BPs that are adsorbed to the bone have a long half-life and are eliminated only when the bone is resorbed [40]. The short half-life in the blood and high affinity to the bone does not allow N-BPs to accumulate in tumours at concentrations necessary for therapeutic efficacy, either as a monotherapy or for use in γδ T cell immunotherapy. Nanoformulations of N-BPs aim to lower bone affinity and increase half-life in the blood circulation [41] and can dramatically change the biodistribution and pharmacological profile of N-BPs.

2. Liposomes

2.1 Introduction to liposomes

Liposomes, a closed bilayer phospholipid system, were first described in 1965 [42]. They are defined as “phospholipid vesicles consisting of one or more concentric lipid bilayers enclosing discrete aqueous spaces” [43]. Liposomes have since been developed as a drug delivery system for many different therapeutic applications including delivery of cancer

chemotherapy, vaccines, gene therapy, antimicrobials, biomolecules and topical drug delivery as well as for use in diagnostic techniques [44]. Liposomes have been shown to overcome barriers to cellular and tissue uptake and improve stability and biodistribution profiles of therapeutic agents *in vivo* [43].

Liposomes were proposed as drug carriers in cancer therapy due to their ability to be preferentially taken up in tumours [45] and have been shown to enhance the efficacy and safety of chemotherapeutic agents [46]. Tumour vasculature inside tumours undergoing angiogenesis tend to exhibit leaky endothelial lining as rapid tumour growth leads to structural abnormalities. This results in blood vessels that are permeable to nanoparticles such as liposomes. This effect is further reinforced by the lack of efficient lymphatic drainage of the tumour which causes liposomes to accumulate preferentially in the tumour area. This is known as enhanced permeation and retention effect (EPR) as illustrated in **Figure 2** [47]. Particles of 10 – 500 nm are thought to be able to extravasate into tumours as the pore sizes in the endothelial lining of leaky blood vessels in peripheral tumours are estimated to be 400 – 600 nm in diameter [48]. However, particles with diameters less than 200 nm have been shown to be more effective at accumulating in tumour sites. This passive tumour targeting does not occur in all tumours and vessel leakiness may also be heterogeneous within a single tumour [49]. Ligand-targeted or ‘active’ targeting of liposomes may result in liposomes that are more selective to cancer cells, once passive targeting has taken place [46].

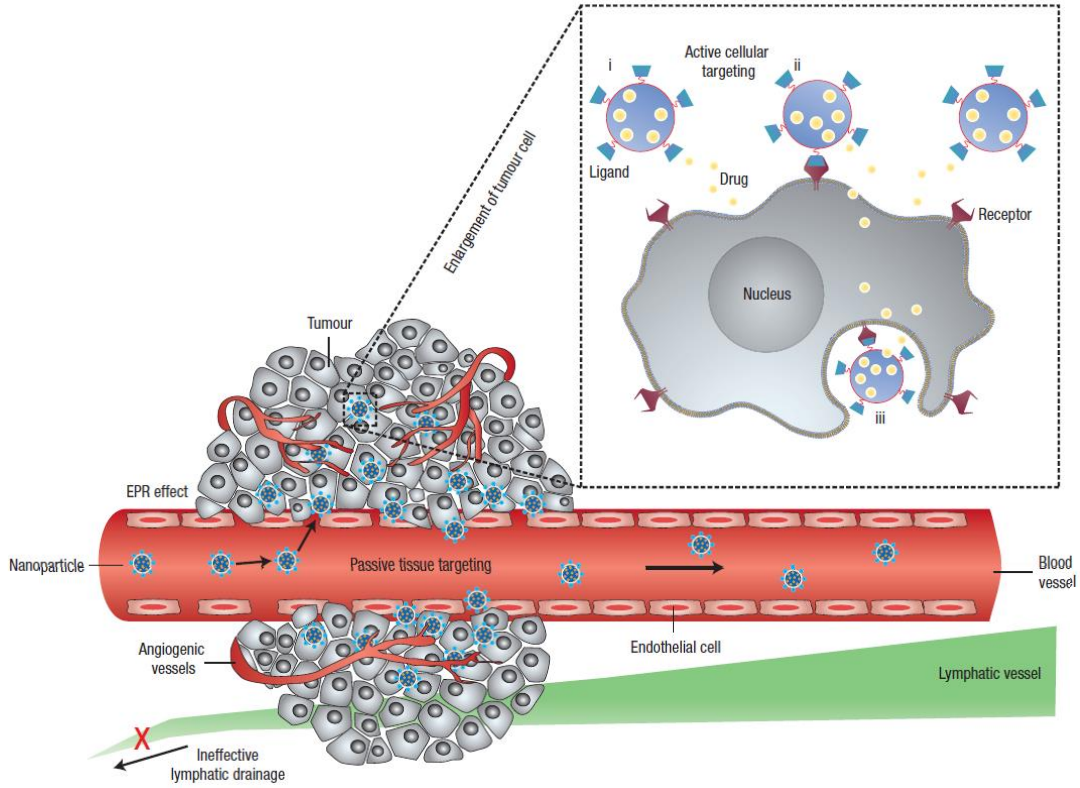

Figure 2: The enhanced permeation and retention (EPR) effect. Tumour vasculature has increased permeability compared to healthy blood vessels. Extravasation of nanoparticles through increased pores in vessel walls coupled with ineffective lymphatic drainage results in enhanced permeation and retention of the particles in tumours (EPR effect). Nanoparticles can be functionalised with ligands in active cellular targeting. The nanoparticles can (i) release their contents in close proximity to the target cells; (ii) attach to the membrane of the cell and act as an extracellular sustained-release drug depot; or (iii) internalise into the cell (adapted from Peer et al., 2007 [47]).

Liposomes can be used to increase the therapeutic index of a drug whereby a greater amount of the active drug reaches the tumour cell leading to an increased cytotoxic effect while at the same time side effects are reduced because of drug encapsulation [50].

2.2 ZOL nanoformulations

Many different nanoformulations of ZOL have been formulated and their *in vitro* and *in vivo* activities have been examined. A summary of studies carried out using non-liposomes nanoformulations of ZOL for cancer therapeutics are described in **Table 1**. Studies using liposome formulations of ZOL are described in more detail below.

A stealth liposome formulation of ZOL (LipoZOL) was shown to enhance delivery of ZOL to extra-skeletal sites through the EPR effect [41]. LipoZOL (Formulation 1) composed of Egg PC, DSPE-PEG2000 and cholesterol was compared to free ZOL both *in vitro* and *in vivo*. *In vitro*, LipoZOL showed lower IC_{50} values compared to free ZOL for 15 of the 17 cancer cell lines tested. CD-1 athymic (nu/nu) mice bearing PC3 xenografts were treated with 10 or 20 µg of ZOL intravenously in its free form or as LipoZOL-PEG three times a week for three weeks. These experiments revealed tumour reductions of 16% and 22% following treatment with 10 and 20 µg respectively of free ZOL compared to tumour inhibition of 58% and 68% when treated with 10 or 20 µg of ZOL administered as LipoZOL-PEG. [41].

A hybrid nanoparticle-liposome formulation (Formulation 2) has also been prepared consisting of a calcium phosphate core and an outer phospholipid bilayer (PLCaPZ NPs) [51]. Calcium phosphate nanoparticles (CaP NPs) were prepared to which ZOL could bind. These CaPZ NPs were then mixed with DOTAP/chol/DSPE-PEG_{2000} liposomes. The PLCaPZ NPs had lower IC_{50} values than free ZOL in all cancer cell lines tested including prostate, breast, head/neck, lung, pancreas and multiple myeloma cell lines [52]. *In vivo* studies were performed using immunosuppressed mice bearing PC-3 tumours. PLCaPZ NPs achieved a significant tumour weight inhibition of 45 % [53]. It is thought that when the hybrid nanoparticle is endocytosed into the cancer cell, the decreasing pH of the endosome causes the CaP to dissolve and the subsequent increase in osmotic pressure across the endosomal membrane leads to the disorganisation of the endosome, allowing the ZOL to be released into the cytosol [51]. The PLCaPZ nanoparticles were also compared to ZOL-containing stealth liposomes (LipoZOL-PEG). The stealth liposomes had significantly lower encapsulation efficacy compared to the PLCaPZ NPs (5.6% *vs.* 66%). Both formulations achieved an inhibition in tumour weight; PLCaPZ NPs showed a 52% reduction of tumour

burden while LipoZOL-PEG caused a reduction of 28%. Tumour growth delay was also seen in the case of PLCaPZ NPs (12 days) and for LipoZOL-PEG (7 days) compared to the untreated group [52].

ZOL containing liposomes have also been modified to obtain a targeted drug delivery system. Shmeeda *et al.* prepared ZOL formulations with or without 0.5 % molar ratio folate-PEG (3350)-DSPE. *In vitro* toxicity of the formulations as well as free ZOL after 72 hours was evaluated on various cell lines. It was shown that both targeted and non-targeted formulations had little toxicity against low folate receptor (FR)-expressing normal human fibroblasts and J774 macrophages. However while the untargeted formulation had an IC_{50} value of more than 200 μM for the high FR expressing KB cell line, the folate targeted version had an IC_{50} value of 0.4 μM. Similarly, a human ovarian cancer cell line IGROV-1 which naturally overexpresses FR had an IC_{50} of 0.1 μM which is significantly lower than the IC_{50} obtained from non-targeted L-ZOL (IC_{50}=50μM) or free ZOL (IC_{50}=35 μM). Drug uptake was examined using a radioactive zoledronic acid tracer (C14-ZOL) and shown to be 50 and 25 times higher for targeted liposomes compared to the non-targeted liposomes and the free ZOL, respectively [54].

However, because the liposomal ZOL has a different biodistribution profile to free ZOL, an increase in systemic toxicity was seen during an *in vivo* studies in mice [55]. In contrast to the decrease in *in vivo* toxicity observed with the liposomal encapsulation of other cytotoxic drugs [56], the liposomal formulations of ZOL showed a large increase in systemic toxicity compared to the free drug. This toxicity was also observed when liposomal ZOL was used with γδ T cells, as previously mentioned [57]. C-14 ZOL and H3-Chol was used to track the ZOL and the liposomes *in vivo* [55].The liposomal formulations showed half-lives of 9-18

hours while free ZOL was almost totally cleared from the plasma within one hour. KB or IGROV-1 tumour bearing mice were treated with free ZOL or a ZOL liposome formulation, with mice receiving 30 μg ZOL in each case. Mice injected with 30 μg of ZOL in the liposome formulations died 5-7 days after injection whereas only occasional deaths (10-20%) were observed with 100 μg of free ZOL. Higher levels of ZOL were found in the spleen, liver, lung and skin when the liposomal formulations were given. Splenomegaly, leukocytosis and thrombocytopenia were observed in the mice injected with ZOL liposome formulations. No overt signs of toxicity were seen when the mouse organs were histopathologically examined. Tumour cells, endothelial cells, tumour-associated macrophages and other tumour-infiltrating cells were exposed to higher concentrations of drugs than if the free drug was given. It was found that a co-injection of a large amount of blank liposomes with the ZOL liposome formulations reduced the systemic toxicity and enabled higher doses of ZOL liposomes to be injected. This indicates that macrophage uptake was involved in the mechanism of systemic toxicity. Low drug:lipid ratios also lowered the toxicity of the ZOL liposomes [55]. Because of this, it is suggested that the systemic toxicity may be due to macrophage activation that leads to a cytokine storm [55]. This hypothesis is supported by previous *in vivo* work with free ZOL [58].

Table 1: Cancer therapy studies using non-liposomal ZOL nanoformulations

Study	Formulation	Composition of formulation	Experimental Parameters	Cell Line	Findings
Benyettou et al, 2009 [59]	γFe_2O_3@ZOL	ZOL and γFe_2O_3 nanocrystals	Nanoparticles incubated with cells for 48 and 72 h at various concentrations with or without a magnetic field	MDA-MB-231	75% proliferation decrease with magnetic field and 40% without at 100µmol L^{-1}
Agrati et al, 2011[60]	ZOL niosomes	Tween 20-Chol-DCP (dicetyl phosphate) with or without ε-PLL (L-lysine polymer)	Niosomes were incubated with leukocytes and γδ T cell activation was monitored by analysing IFN-γ production.	Human leukocytes and lympho-monocytes from healthy donors	29.7-48.3% of the γδ T cells were activated by the ZOL loaded noisome clusters
Liu et al, 2012 [61]	Nanoscale Coordination polymer with ZOL	$CaCl_2 \cdot 2H_2O$ was loaded with ZOL and surrounded by DOTAP/ DOPE (1:1 mol) liposomes	Formulations were incubated with cell lines and cell apoptosis was measured by Annexin V-FITC	H460 cells and AsPC-1	IC_{50} values of 1.0 ± 0.5 µM with H460 cells and 3.6 ± 2.3 µM for AsPC-1 cells
Chen et al, 2013 [62]	Lipid coated calcium phosphate nanoparticles containing ZOL and double-stranded RNA (poly (I:C))	Calcium phosphate, poly (I:C), zoledronate, DOTAP and cholesterol	Nanoparticles incubated with cells for 48 h and cytotoxicity assessed by MTT assay. Animal studies carried out in female C57BL/6 mice. Formulations given to result in 4.5µg ZOL per mouse	Mouse melanoma cell line B16BL6	Nanoparticles containing poly (I:C) and ZOL had a cell viability of 14.4% Nanoparticles containing poly (I:C) and ZOL resulted in significant tumour growth inhibition ($p<0.05$)

2.2 ALD nanoformulations

ALD has been frequently used as a targeting moiety on nanoparticles for delivery of therapeutic agents to the bone [63-67]. However, it has also been encapsulated into nanoparticles for its anti-cancer and other pharmacological activities. ALD has also been co-encapsulated with doxorubicin (DOX) into liposomes [68]. Liposomes encapsulating both drugs (PLAD) were shown to be superior to liposomes encapsulating DOX alone (PLD). PLAD was shown to be more cytotoxic than PLD in several cell lines tested *in vitro*. PLAD was also shown to be more effective than PLD at inhibiting tumour growth in 4T1 breast cancer and M109R lung cancer models in BALB/c mice *in vivo*. ALD has also been encapsulated in nanoformulations other than liposomes for use in anti-cancer therapy. These studies are summarised in **Table 2**.

Liposomal ALD has also been studied for its ability to deplete macrophages. ALD liposomes have been shown to inhibit macrophages *in vitro* and deplete circulating monocytes in rabbits *in vivo* [69]. This anti-inflammatory activity of ALD liposomes has shown to be effective in the inhibition of restenosis (the recurrence of narrowing of an artery following corrective treatment, for example, by angioplasty or stent) in rabbits *in vivo* [69]. Negatively-charged ALD liposomes have been found to be more potent inhibitors of monocytes and macrophages than neutral ALD liposomes *in vitro* [70]. Liposomal ALD was also shown to cause cytokine activation of human blood *ex vivo*, with increased levels of IL-1β, TNF-α, IFN-γ, IL-5, IL-6, IL-8, IL-10 and IL-12p70. *In vivo* secretion of IL-1β was also observed but there was no complement activation seen [70]. The ability of ALD liposomes to deplete monocytes and macrophages has also been shown to inhibit restenosis and endometriosis in a rat model [71]. However, while both IP and IV administration of ALD liposomes were effective in restenosis inhibition, only IP injections were effective in the treatment of endometriosis. ALD

liposomes have also been shown to deplete monocytes and macrophages in non-human primate model of human disease *in vivo* [72]. At doses of 0.1 mg/kg, a more than 50 % decrease in levels of circulating monocytes and tissue-resident macrophages was observed. The treatment was also shown to be well tolerated with no adverse clinical side effects observed. A clinical trial to evaluate the use of ALD in the prevention of coronary artery restenosis is due to commence [73].

Table 2: Cancer therapy studies using non-liposomal ALD nanoformulations

Study	Formulation	Composition of formulation	Experimental Parameters	Cell Line	Findings
Dolatabadi et al., 2014 [74]	Solid lipid nanoparticles	Solid lipid Precirol® ATO 5 or Compritol 888 ATO® with Tween 20 and Poloxamer 407 as surfactants	Particles incubated with cells for 24 h prior to MTT assay	A549 lung carcinoma cells	ALD solid lipid nanoparticles did not have significant toxicity
Zhan et al., 2014 [75]	Glucomannan conjugates	Glucomannan polysaccharide conjugated to ALD	Conjugates incubated with cells for 24 h prior to MTT assay. Cell cycle and cell apoptosis measured with flow cytometry S180 tumour bearing mice treated with 10 mg/kg free or conjugated ALD	Mouse macrophage cell line Raw 264.7, mouse sarcoma cells S180, HUVECs and human lung carcinoma A549	Viability of cells reduced *in vitro* treated with free of conjugated ALD Conjugated ALD significantly inhibited increase in tumour size and eliminate tumour associated macrophages
Zhu et al., 2014 [76]	Nanoparticles	Zirconium based metal-organic frameworks of UiO-66 conjugated with ALD	UiO-66 and ALD nanoparticles incubated with cells for 24 and 48 h	HepG2 liver carcinoma cells and MCF-7 breast cancer cells	After 48 h incubation, the UiO-66 and ALD nanoparticles led to higher cytotoxicity than free ALD
Massey et al., 2016 [77]	Nanoparticles	Alendronate was complexed with the cationic amphiphilic peptide RALA to spontaneously form nanoparticles	Particles were incubated with cells for 6 h and cells were incubated with fresh media for 72 h prior to MTS assay. PC-3 tumour bearing BALB/c SCID treated with 10 µg free ALD or ALD in particles	PC3 prostate cancer cells and MDA-MB-231 breast cancer cells	Complexation with RALA potentiated the cytotoxic effects of ALD *in vitro.* Significant growth delay of tumours and increase in mean survival time *in vivo*

3.Gamma Delta T cells

3.1 Functions of gamma delta T cells

N-BPs can be used in cancer immunotherapy due to their ability to activate gamma delta (γδ) T cells [4]. γδ T cells are a subset of human T lymphocytes. They develop in the thymus with αβ T cells but have a rearranged T cell receptor (TCR) consisting of a TCR-γ and a TCR-δ chain [78]. γδ T cells can also be grouped according to tissue location or by the variable segments of the γ and δ TCR chains. They represent 1-10% of all peripheral blood T cells [79] and can be found widely in the skin, intestines and reproductive tract where they can make up to 50 % of T cells [80]. They are also present in the liver, spleen and thymus [81]. There are three main Vδ chains (Vδ1, Vδ2 and Vδ3) and seven main Vγ chains (Vγ2, Vγ3, Vγ4, Vγ5, Vγ8, Vγ9 and Vγ11) [82]. In humans, peripheral blood γδ T cells express mainly the Vγ9Vδ2 T Cell Receptor (TCR) [83], while in peripheral organs Vδ1 and Vδ3 are the predominant variant present [81]. Peripheral blood γδ T cells can proliferate to make up to 50% of total peripheral T lymphocytes when stimulated and that this expansion can last up to four months following some microbial infections [84, 85]. Due to the heterogeneity of their TCRs and the fact that they are not major histocompatibility complex (MHC) restricted, γδ T cells have a diverse range of roles in the immune system (**Figure 3**). They are implicated in the immune response to infectious diseases, autoimmune disease and tumour surveillance [80]. They are associated with the innate function of the immune system as they have a rapid cytokine response and mainly reside in mucosal tissues [86]. They share effector functions with alpha beta (αβ) T cells and natural killer (NK) cells [87, 88]. γδ T cells have also been shown to share some properties with antigen-presenting cells such as dendritic cells [89] and may have an immunoregulatory role [90].

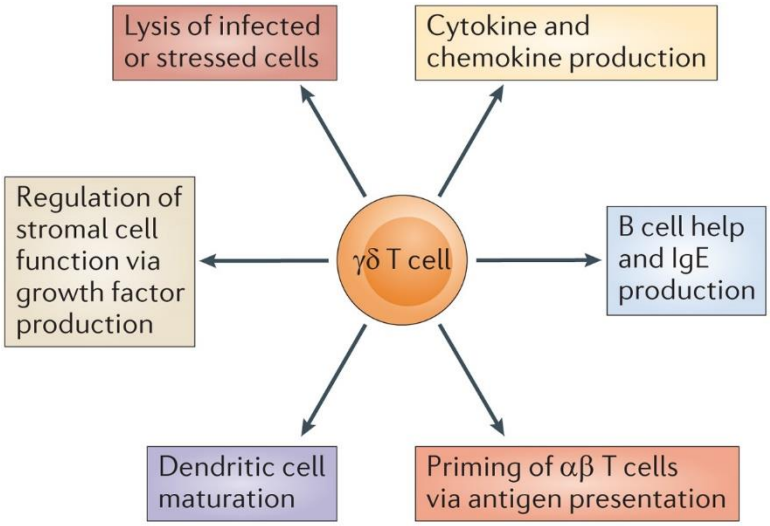

Figure 3: Illustration of the role of γδ T cells in the immune system. γδ T cells have been shown to have a wide range of roles in the immune system through six main mechanisms; (1) γδ T cells can produce granzymes to directly lyse infected or stressed cells, (2) they secrete a range of cytokines and chemokines which regulate both immune and non-immune cells; (3) they assist B cells and promote the production of IgE, (4) they can present antigens to αβ T cells, (5) they can trigger dendritic cell maturation and (6) they produce growth factors to regulate stromal cell function (Adopted from Pierre Vantourout & Adrian Hayday, 2013 [91]).

When activated γδ T cells secrete perforin and granzymes (cytoplasmic granule toxins) as well as cytokines such as IFN-γ and tumour necrosis factor (TNF)-α. Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) expression by γδ T cells is also up-regulated [92]. Some γδ T cells also express CD16 (the low affinity Fc receptor for IgG) which can bind to anti-tumour cell monoclonal antibodies, thereby promoting antibody-dependent cellular cytotoxicity [83]. They can also induce NK cell-mediated cytotoxicity through the CD137 pathway [93]. A subset of γδ T cells which have low or no CD27 surface expression produce IL-17 [94]. Some of the multiple functions of γδ T cells are displayed in **Figure 4**.

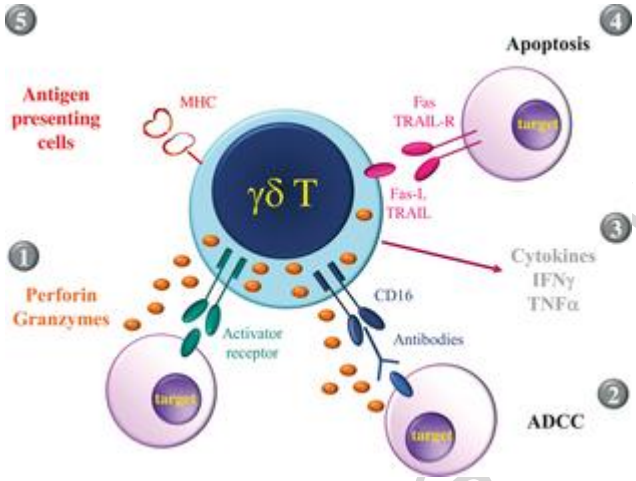

Figure 4: Multiple functions of γδ T cells. (1) Following T cell receptor (TCR)-dependent activation, γδ T cells release perforin and granzymes, as an early step of their cytotoxic activity. (2) $CD16^{+}$ effector γδ T cells bind to the Fc region of monoclonal antibodies that target tumour cells, thereby exercising their antibody-dependent cellular cytotoxicity (ADCC) function. (3) γ interferon (INFγ) and tumour necrosis factor α (TNFα) secretion by γδ T cells indicate their implication in immune defence/response networks. (4) Upon TCR engagement, Fas ligand (Fas-L) and TNF-related apoptosis-inducing ligand (TRAIL) expression is up-regulated in γδ T cells and as, a consequence, the Fas- or TRAIL-receptor (R) sensitive tumour cell killing by γδ T cells is enhanced. (5) Human γδ T cells can act as professional antigen-presenting cells because they can process and display antigens and can also provide co-stimulatory signals necessary for the induction of proliferation, differentiation and target cell killing. MHC, major histocompatibility complex. (Adopted from Braza *et al*, 2013 [95]).

3.2 γδ T cell activators

γδ T cells recognise a diverse range of antigens. While γδ T cells can recognise antigens in a non-MHC dependant manner, MHC molecules have also been shown to be ligands for the γδ TCR [96] such as MHC class I polypeptide related sequence (MIC) A, MICB and UL16 binding protein (ULBP) which activate γδ T cells [85, 97]. Soluble proteins such as the tetanus toxoid [98], bacterial proteins [99], viral proteins [100] and heat shock proteins (HSP) [101] can stimulate γδ T cells. It has also been shown that γδ T cells can recognise cell-surface expressed proteins other than MHC molecules [102]. Human γδ T cells also recognise group 1 CD1 molecules that are mainly found on professional antigen presenting cells and present lipid antigens such as glycolipids and microbial lipids [103]. Additionally, γδ T cells have been shown to respond to peptides without requiring antigen presenting cells [104]. γδ T

cells have high levels of expression of the IL-2 receptor and this cytokine is necessary for their survival and proliferation [105].

γδ T cells that express the Vγ9Vδ2 TCR are activated by phosphorylated antigens in a MHC-independent manner [95] without the need to process and present these antigens [106]. These antigens are usually natural metabolites known as natural phospho-antigens (PAgs), for example, isopentyl pyrophosphate (IPP). These PAgs are produced by many different microorganisms such as *Escherichia coli*, *Mycobacterium tuberculosis* and *Plasmodium falciparum* [107]. They are also overproduced in dysregulation of the mevalonate pathway in human cells, such as in the case of tumour cells [108]. When activated by the PAgs, γδ T cells activate innate immune cells and are also directly involved in the elimination of some pathogens or tumour cells [95]. Cell-to-cell contact has been shown to be necessary to achieve γδ T cell activation [109]. While the PAgs are recognised in a TCR-dependent manner, it is thought that the PAgs may induce the structural modification of the TCR [110]. The PAgs bind to the intracellular B30.2 domain of the butyrophilin 3A1 (BTN3A1) protein [111]. BTN3A1 is a member of the BTN3A family, also known as CD277 which have been shown to have immunoregulatory functions [112]. It is hypothesised that binding of a ligand to the B30.2 domain leads to a conformational change which then modulates the extracellular domains of BTN3A1. It is not known however whether BTN3A1 can directly activate the Vγ9Vδ2 TCR, or if other as yet unknown proteins may be recruited in order to do this [82]. RhoB has been shown to be a critical mediator in Vγ9Vδ2 activation [113].

Nitrogen containing bisphosphonates (N-BPs) are a class of drug that inhibit FPP synthase and lead to intracellular accumulation of PAgs such as IPP and triphosphoric acid I-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) ester (ApppI) [114] as shown in **Figure 5**. Conversely,

statins reduce IPP accumulation by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) of the mevalonate pathway and therefore reducing γδ T cell activation [4]. N-BPs can also inhibit γδ T cell proliferation upon continuous exposure both *in vivo* and during *ex vivo* expansion due to blocking of isoprenoid metabolism in γδ T cells leading to decreased levels of farnesyl pyrophosphate and geranylgeranyl pyrophosphate. This results in decreased cell proliferation and survival. An alternative method of pulsing γδ T cells with N-BPs results in good *ex vivo* expansions with reduced toxicity to the γδ T cells compared with continuous exposure [115]. Alkylamines also act as indirect stimulators of γδ T cells and cause the accumulation of PAgs by inhibition of FPP synthase in the same manner as N-BPs, however at much lower potency [116]. Mevalonate has also been shown to stimulate γδ T cells, though not to the same extent as the N-BPs. High exogenous mevalonate concentrations bypass normal regulation of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) and increases the levels of downstream products such as IPP. This stimulation cannot be blocked by statins [115]. A mushroom extract, Polysaccharide K, has been shown to activate γδ T cells which may partly account for its *in vivo* anti-tumour and immunomodulatory effects [117].

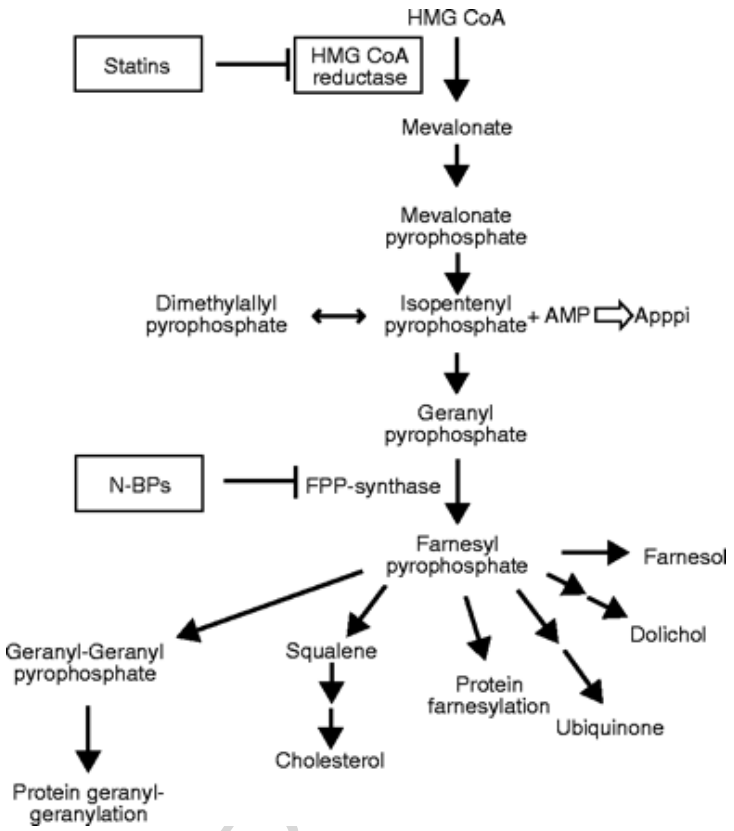

Figure 5: Schematic representation of the mevalonate pathway and the effects of nitrogen-containing bisphosphonates. Nitrogen-containing bisphosphonates block the enzyme Farnesyl pyrophosphate synthase (FPP-synthase) preventing the prenylation of proteins and leading to the upstream accumulation of Appp1 and phosphoantigens such as Isopentenyl pyrophosphate (IPP). (Adapted from www.ufrgs.br)

γδ T cells have also been shown to be activated by antibodies. Bispecific antibodies that bind CD3 or Vγ9 on γδ T cells and Her2/neu on pancreatic tumour cells have been developed [118]. Both the Her2/CD3 and Her2/Vγ9 antibodies resulted in enhanced γδ T cell cytotoxicity *in vitro*. Additionally, the Her2/Vγ9 antibody led to an enhanced release of perforin and granzymes. When pancreatic tumour bearing SCID/beige mice were treated with γδ T cells and the Her2/Vγ9 antibody, a reduction in tumour growth was observed. Vγ9Vδ2 T cells have also been specifically activated by nanobodies [119] which consists of a single immunoglobulin domain of the variable antigen binding region of non-conventional heavy chain only antibodies.

3.3 γδ T cells and cancer

Vγ9Vδ2 T cells naturally recognise tumour cells through recognition of PAgs accumulated intracellularly [4] or interaction with cell surface proteins such as F1-ATPase [102]. A mutation in *p53*, present in more than 50 % of human cancers, has been shown to significantly upregulate the mevalonate pathway [120]. Other transformation induced changes of cell surface markers can enhance tumour immunogenicity and lead to recognition by Vγ9Vδ2 T cells [121]. The activation of Vγ9Vδ2 T cells by tumour cells is illustrated in **Figure 6**. They are thought to have an immunosurveillance role in detecting malignancies [106] and can also migrate into solid tumours as infiltrating lymphoctyes [122]. Infiltration of γδ T cells has been reported in many different tumour types including melanoma, ovary, colon, lung and prostate [123]. In melanoma patients, tumour infiltrating γδ T cells were significantly associated with lower mortality and relapse rates [124]. Additionally, patients who progressed to stage III or IV melanoma had a significantly decreased frequency of circulating Vγ9Vδ2 T cells. The presence of γδ T cells in tumour infiltrating lymphocytes (TIL) has also been shown to result in lower metastatic spread and a longer mean survival time [125, 126]. *In vitro*, Vγ9Vδ2 T cells that have been isolated from ascites of a colon cancer patient have been shown to be cytotoxic when cultured with colorectal cancer cell lines [126, 127]. However, the immunosuppressive microenvironment of the tumour can limit the effectiveness of the anti-tumour activity of γδ T cells [128].

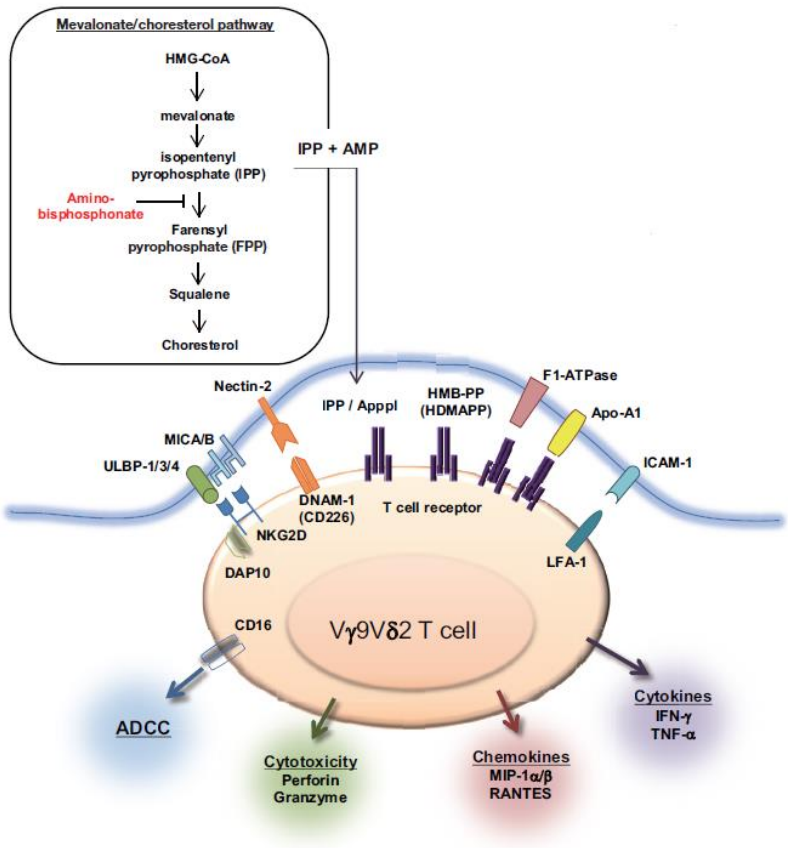

Figure 6: Recognition of tumour cells by human γδ T cells. N-BPs block the mevalonate pathway in mammalian cells leading to increased intracellular IPP levels. IPP metabolites can also be converted into ApppI. Both IPP and ApppI can be presented at the cell surface and are recognised by the γδ TCR. γδ T cells also recognise MHC molecules such as MICA, MICB and ULBP as well as other cell-surface expressed proteins (Adapted from Kakimi et al, 2014 [121]).

Some cytotoxic compounds can trigger 'immunogenic' cell death including doxorubicin and oxaliplatin. These chemotherapeutic agents act on tumour cells in such a way that the host immune system recognises the dying tumour cell. A tumour-specific immune response occurs during cell death which results in an anti-tumour immune response leading to tumour eradication and prevention of relapse. This immunogenic cell death can prevent immune tolerance to tumour cells and is a crucial component of treatment efficacy [129]. γδ T cells can be recruited to a tumour after exposure to immunogenic chemotherapy and can contribute to the efficacy of the chemotherapy [130]. *In vitro*, pre-treatment with low concentrations of chemotherapeutic agents (doxorubicin, cisplatin, etoposide and vincristine) or ZOL have been

shown to sensitise tumour cells to killing by γδ T cells with additive or synergistic effects. [131].

There are two main approaches to γδ T cell cancer immunotherapy; the *in vivo* expansion of γδ T cells by administration of compounds that activate them or the adoptive transfer of *ex vivo* activated γδ T cells. Many studies have been performed using one of these strategies as detailed below.

3.4 *In vivo* expansion of γδ T cells

Many clinical trials involving the *in vivo* activation of γδ T cells have been published in the literature for a wide variety of cancers as seen in **Table 3**. These trials are based on the co-administration of N-BPs or other PAgs with IL-2 to patients, which have been shown to increase the number of circulating γδ T cells [132]. *In vivo* activation of γδ T cells does not occur in all patients however. For example, ten patients with Non-Hodgkin's Lymphoma (NHL) of the B-cell type received 90 mg of pamidronate (PAM) followed by 0.25-3 million IU/m^2 of IL-2 daily for six consecutive days. None of these patients showed a response to treatment and no activation or expansion of their γδ T cells was observed [133]. However, when nine patients were pre-selected on the basis of an *in vitro* γδ T cell response to PAM/IL-2 and the same treatment was given, 55% of patients showed a statistically significant increase in γδ T cell number *in vivo* and partial remission was seen in 33% of patients (indicated by >50% reduction for all measureable lymphoma manifestations and no new lesions for at least four weeks) [133]. The importance of IL-2 in the *in vivo* expansion of γδ T cells has been observed. A study conducted by Dieli *et al* attempted to induce activation of γδ T cells *in vivo* using ZOL alone or in combination with low-dose IL-2 in patients with metastatic hormone-refractory prostate cancer (HRPC). No expansion of γδ T cells were seen

in the group treated with ZOL alone while in the group treated with ZOL and IL-2, five out of nine patients showed an increase in γδ T cell population. In the group treated with ZOL alone one patient showed stable disease over 14 months and one showed partial remission (30% or more reduction or the longest diameter of all measureable lesions). In contrast, six out of the nine patients in the group treated with ZOL and IL-2 showed good clinical responses with four patients achieving stable disease for 14-16 months and two patients showing a partial remission [134].

Table 3: Human studies of *in vivo* expansion of γδ T cells

Study	Carcinoma	Dosing Regimen	Effect on γδ T cells	Clinical Outcomes
Wilhelm et al. 2003 [133]	NHL of the B-type	90mg PAM once and 0.25-3 million IU/m^2 IL-2 for 8 days	Significant proliferation in 55% of patients	33% partial response (more than 50% reduction in manifestation), 22% disease stabilisation (less than 50% reduction in manifestations).
Dieli et al, 2007 [134]	Metastatic hormone refractory prostate cancer	ZOL with or without IL-2. (Dose and frequency varied amongst patients)	Increase in γδ T cell response seen	Disease stabilisation or partial remission †
Bennouna et al, 2010 [135]	Solid tumours	BrHPP and IL-2. (Dose and frequency varied amongst patients).	Strong amplification of γδ T cell in 39 of 45 patients	43% of patients had stable disease †
Meraviglia et al, 2010 [136]	Metastatic breast cancer	4mg ZOL and 1 x 10^6 IU IL-2 every 21 days over a one year period.	The majority of patients saw a decrease in the number of γδ T cells when compared to pre-treatment levels.	10% of patients had a partial response and 20% stable disease †
Lang et al, 2011 [137]	Metastatic renal cell carcinoma	4mg ZOL weekly for three weeks with varying IL-2 dosing regimens	Decrease in γδ T cell response	No significant clinical effect seen †

† Based on RECIST (Response Evaluation Criteria in Solid Tumour) criteria [138].

3.5 *Ex vivo* expansion of γδ T cells

γδ T cells that have been expanded *ex vivo* and are subsequently administered to cancer patients have also shown promising anti-tumour activity as summarised in **Table 4**. In these studies, γδ T cells are isolated from a patient and expanded *ex vivo*, typically in the presence of IL-2 and ZOL, for a period of time before being infused back into the patient [139]. Advanced renal cell carcinoma patients' γδ T cells were expanded from PBMC over a two week period with IL-2 and the phosphoantigen, 2-methyl-3-butenyl-1-pyrophosphate (2M3B1-PP) before being re-infused to the patient. Three of the seven patients showed slower tumour growth [140]. γδ T cells have also been isolated from the tumour infiltration lymphocytes (TIL) of 15 non-small cell lung cancer patients and were expanded *ex vivo* for two weeks in the presence of ZOL and IL-2 before re-infusion. Six patients experienced brief disease stabilisation and there was an increase in mean progression free survival overall survival [141].

Immunomonitoring whereby γδ T cells were labelled with indium 111 (In^{111}) and tracked in patients have shown that cells are trafficked mainly to the lungs where they remain for 4-7 hours before migrating to the liver and spleen in all patients [142] with only a minority of the γδ T cells reaching metastatic tumour sites. The γδ T cells were seen at tumour sites 1 hour after injection with maximum levels at 4 hours. Despite the small percentage of γδ T cells reaching tumour sites, several of the patients exhibited disease stabilisation or partial response; with one patient having a complete response. The number of γδ T cells that can reach the tumour site may limit the clinical efficacy of this treatment [132].

A limitation to the use of autologous γδ T cells is the frequent impaired function of γδ T cells in cancer patients. The reason for this γδ T cells anergy has not been fully elucidated but

regulatory T cells have been shown to inhibit phosphoantigen-induced proliferation of γδ T cells [143]. This has also been observed in certain chronic infectious diseases such as HIV and tuberculosis [144]. As γδ T cells do not cause graft-versus-host disease (GVHD), the allogeneic transfer of γδ T cells is a viable option for immunotherapy [145]. Patients with advanced haematological malignancies received γδ T cells from half-matched family donors in addition to ZOL and IL-2 [146]. Three out of four patients treated achieved a complete remission with no signs of GVHD observed.

Table 4: Human studies of *ex vivo* expansion of γδ T cells

Study	Carcinoma	Dosing Regimen	Effect on γδ T cells	Clinical Outcomes
Kobayashi et al, 2007 [140]	Advanced renal cell carcinoma	Autologous γδ T cells and 0.7 million IU IL-2 per patient weekly 6-12 times	Significant in vivo expansion and production of IFN-gamma	42% of patients had slower tumour growth †
Bennouna et al, 2008 [135]	Metastatic renal cell carcinoma	γδ T cells (Innate Pharma) given every 21 days for three cycles with 2 x 10^6 IU/m^2/day IL-2 for 7 days after the 2nd and 3rd γδ T cell infusions	γδ T cells increased substantially after the 2nd and 3rd infusions	60% of patients showed stabilised disease ‡
Abe et al, 2009 [147]	Multiple myeloma	Autologous γδ T cells given every 2 weeks for 4 cycles	γδ T cells percentage in the PBMC increased substantially	No significant clinical response seen.
Nakajima et al, 2010 [148]	Non-small cell lung cancer	Autologous γδ T cells given every 2 weeks for 3-12 cycles	γδ T cells were shown to survive more than two weeks *in vivo* after infusion	No complete response or stabilisation observed. Stable or improved 'functional assessment of cancer-therapy-biologic response modifier scores' ‡
Kobayashi et al, 2011 [149]	Lung metastasis from renal cell carcinoma (one patient)	4mg ZOL, 1.4 million IU IL-2 and 0.3-3.5 x 10^9 autologous γδ T cells monthly for 6 months	Significant *in vivo* γδ T cell proliferation and IFN-gamma production was seen.	Complete remission of patient, currently ongoing two years after study with no further treatment ‡
Noguchi et al, 2011 [150]	Solid tumours	Autologous γδ T cells given 3-6 times	All but three patients showed amplification of γδ T cells >10^8	6 out of 25 patients experienced disease stabilisation ‡
Nicol et al, 2011 [142]	Advanced stage with various solid tumours	Autologous γδ T cells given 6-8 times	Radiolabelled γδ T cells were tracked to metastatic tumour sites	3 out of 18 patients had stabilised disease, 2 patients had partial response and 1 patient had a complete response ‡

Sakamoto et al, 2011 [141]	Recurrent or advanced non-small cell lung cancer	Autologous γδ T cells given every 2 weeks for 6 cycles	The number of peripheral γδ T cells increased after repeated infusions.	50% of patients had stabilised disease ‡
Takamichi et al, 2013 [151]	Colorectal cancer	Autologous γδ T cells ($1.2 \pm 0.6 \times 10^6$/kg) given weekly for 8 weeks	The number of peripheral γδ T cells increased after repeated infusions and were functionally active	Not reported
Wilhelm et al, 2014 [146]	Advanced haematological malignancies	2.17×10^6/kg (range 0.9-3.48) allogeneic γδ T cells, 4 mg ZOL and 1.0×10^6 IU/m^2 IL-2	Mean 68 fold *in vivo* expansion of γδ T cells	3 out of 4 patients had complete remission ‡

† Based on estimation of prolonged tumour doubling time by CT

‡ Based on RECIST (Response Evaluation Criteria in Solid Tumour) criteria [138].

3.6 Safety and efficacy of γδ T cell therapy

γδ T cell therapy has been shown to be both feasible and safe [132]. In general, this immunotherapy has been well tolerated by patients. Mild adverse reactions such as flu-like symptoms, gastrointestinal disorders, hypotension and tachycardia have been reported. [135]. Some of these side effects are due to the ZOL and IL-2 often injected with the γδ T cells [134]. However, there are remaining issues and limitations to its use in cancer therapy. Some patients are hyporesponsive, whereby their γδ T cells fail to expand as expected, and activation-induced γδ T cell anergy (lack of γδ T cell activation or expansion) has been reported in many studies [132]. The use of allogeneic γδ T cells may overcome this limitation, however.

The ability of γδ T cells to infiltrate tumours may pose a problem and the efficacy of γδ T cells may be limited by how successfully they can infiltrate established tumours [152]. Immunoescape whereby the cancer cells avoid immune system 'checkpoints' and immunoevasion where cancer cells fail to be detected by the immune system or secrete immunosuppressive molecules may also pose problems [132]. Tumours can produce inhibitory factors which can interfere with the proliferation and function of γδ T cells [152]. These include transforming growth factor (TGF)-β [153], prostaglandin-E_2 [154], adenosine [155], soluble NKG2D ligands (such as MICA/B) [156], galectin-3 [157], HLA-G [158] and indoleamine 2,3 dioxygenase (IDO) [159]. There are also several types of suppressive cells in the tumour microenvironment which can inhibit the proliferation and cytotoxic effect of γδ T cells such as regulatory T cells [143], myeloid-derived suppressor cells [140] and mesenchymal stem cells [154].

Genotyping patients for elevated levels of these inhibitory factors and suppressor cells prior to the use of γδ T cell therapy may be helpful in identifying which patients can benefit most from this treatment. Concomitant use of therapies that can overcome immunoescape may also be useful. These include the use of chemotherapeutic drugs that induce immunogenic cell death [129] or non-specific immune stimulation by cytokines such as IL-2 and IFN-α, monoclonal antibodies and other biomolecules such as the anti-CD5 monoclonal antibody or the IL-2-diphtheria toxin conjugate [132]. Novel regimens that combine these drugs with PAgs or with γδ T cells are currently under investigation [132].

3.7 γδ T cell therapy studies carried out in mice

Murine studies have been undertaken to evaluate the effect of N-BPs as sensitising agents for γδ T cell immunotherapy. As mice do not have the Vγ9Vδ2 subset of T cells that are activated by N-BPs and other PAgs, human Vγ9Vδ2 T cells must be used in murine studies. In all studies described below and summarised in **Table 5**, the term γδ T cells refers to cells that have been isolated from human blood samples. In murine models, repeated inoculation with γδ T cells that have been isolated and expanded from human blood has been shown to delay or stop tumour progression in a wide range of cancer types including melanoma, prostate, lung, bladder and breast cancers [160-164]. Several different N-BPs have been used as a pre-treatment prior to the injection of γδ T cells in order to enhance the therapeutic efficacy of the γδ T cell treatments, with ZOL being the most common N-BP used.

It was investigated whether pre-treating mice with ZOL 16 hours prior to injecting γδ T cells would improve survival time of nude/nude athymic mice with SH-SY-5Y neuroblastoma cells adrenal gland tumours [165]. Mice were injected i.v. with 5×10^6 γδ T cells, ZOL (150 μg/Kg), or both, weekly, for 4 weeks. A statistically significant improvement of survival was

observed in mice receiving γδ T cells after ZOL pretreatment, in comparison with untreated mice or. In contrast, survival of mice treated with γδ T cells or ZOL alone was not significantly different from that of the control group. Infiltration of γδ T cells was significantly higher in tumours from mice receiving the combined treatment than when injected with γδ T cells alone. Additionally, IFN-γ production was clearly seen in tumours from mice receiving the combined treatment, whereas it was barely detectable in the tumours from mice receiving γδ T cells alone. SBC-5-Luc tumour-bearing BALB/c *nu/nu* mice treated with 80 mg/kg ZOL 12 h prior to 1 x 10^7 γδ T cells showed similar results [166]. Tumour growth in mice treated with both ZOL and γδ T cells, but not with ZOL or γδ T cells, was significantly lower than tumour growth in untreated mice. γδ T cell and ZOL combinatory treatment has also been shown to result in prolonged survival in a murine orthotopic bladder model [162] compared to mice treated with γδ T cells or ZOL alone.

ALD has also been shown to be an effective sensitiser for γδ T cell immunotherapy [167]. SCID beige mice inoculated i.p. with the melanoma cell line MeWO or the pancreatic cell line PancTu1 were concomitantly treated i.p. with human rIL-2 (300 ng), ALD (10 μg) and varying doses of γδ T cells. Increased mean survival of mice was observed. PAM (50 μg/kg i.v.) has been used 24 h prior to γδ T cells (1×10^6 i.v.) in PC3 tumour-bearing NSG mice [168]. When administered alone, PAM or γδ T cells had no detectable effect on PC3 tumour growth when compared with untreated controls. However, the growth of PC3 tumours was significantly decreased in mice receiving both PAM injection and γδ T cells when compared with other conditions. Immunohistochemical analysis showed the presence of infiltrating γδ T cells only within tumours of NSG mice that received both PAM and γδ T cells. When both PAM and γδ T cells injections were repeated weekly for four cycles, a strong and long-term control of PC3 tumour growth was achieved. Risedronate (RIS) has been used to stimulate

the γδ T cell expansion from human PBMCs implanted intraperitoneally in NOD/SCID mice [169]. Dose dependant stimulation, in the presence of IL-2, up to 50% of human T lymphocytes, was observed versus less than 10% for IL-2 alone. Additionally, a 46% reduction of the volume of T47D tumours was seen in mice treated with PBMC, IL-2 and RIS, when compared with placebo. Similar results were observed when using ZOL in a previous study by the same group [170].

It was attempted to improve the therapeutic efficacy further by encapsulating ZOL in a liposome formulation [57]. However, after observing toxicity when liposomal ZOL was used, therapy studies were unable to be performed using this bisphosphonate. Further experiments were performed using the alternative bisphosphate, alendronic acid (ALD) in intraperitoneal (i.p) models of the ovarian cancer cell lines SKOV-3-luc and IGROV-1-luc in SCID/Beige mice. Mice were injected i.p. with two doses of free or liposomal ALD (30 μg and 100 μg), 48 and 24 h prior to i.p. injection of 2 x 10^7 γδ T cells. Free and liposomal ALD were shown to be equally effective and significant tumour regression was seen in these treatment groups ($p < 0.001$). However, when the free or liposomal ALD was delivered by i.v. injection (150 μg) followed by three i.p. doses of 1 x 10^7 γδ T cells (24, 72 and 120 hours later), liposomal but not free ALD showed significant tumour regression. A study from our group [171], demonstrated significant inhibition of tumour growth in NSG mice with experimental lung metastatic cancer $p < 0.05$ after intravenous treatment of both L-ALD (0.5 μmol ALD/mouse) and γδ T cells (5 x 10^6 cells/mouse). Treatments with L-ALD or γδ T cells alone did not result in a significant delay in tumour growth.

Table 5: γδ T cell and N-BP studies in mice

Name of Study	Type of N-BP	Mouse Strain	Tumour	No. of γδ T cells	Treatment Protocol	Outcomes of Study
Kabelitz et al (2004) [167]	ALD (10 μg)	SCID beige	MeWO or PancTu1 (i.p.)	Varied	Treated i.p. with rIL-2 (300 ng) and 10 μg ALD every 4 days and variable doses of γδ T cells i.p. on day 0, 4, 10, 20 and 30	Mean survival of mice increased from 28.5 to 87.3 days ($p < 0.0001$) in mice inoculated with MeWo and from 23.0 to 48.4 days ($p < 0.0001$) in the case of PancTu1.
Sato et al (2005) [166]	ZOL (80 mg/kg) 12 h prior to γδ T cells	BALB/c *nu/nu*. 10^7	SBC-5-Luc (sc)	1 x 10^7 γδ T cells (i.v.)	Treated weekly for 3 weeks	Significant reduction in tumour growth ($p < 0.05$)
Yuasa et al (2009) [162]	ZOL (100 μl of 5 μM)	Balb/c SCID,	UM-UC-3Luc cells (intravesically administered into bladder cavity)	1 x 10^7 γδ T cells (intravesically administered into bladder cavity)	Treatments were administered for 3 hours on five sequential days.	Photon emissions were significantly lower than the non-treatment groups and prolonged duration of survival with treatment ($p < 0.001$)
Benzaid et al (2012) [169]	RIS (150 μg/kg)	NOD/SCID	T47D or B02 cells (s.c)	3.5 × 10^7 human PBMCs (i.p.)	Every second day for 14 days	46% reduction of the volume of T47D tumours ($p < 0.05$)
Santolaria et al (2013) [168]	PAM (50 μg/kg i.v.) 24 hours prior to γδ T cells	NSG	PC3 (s.c.)	1 × 10^6 γδ T cells i.v.	Once off treatment or weekly for four weeks	Significantly decreased tumour growth after once off treatment (10 mm versus 20 mm in average diameter at week 5; $p < 0.0005$) and a strong and long-term control of tumour growth ($p < 0.0005$) after four treatments.
Di Carlo et al (2013)	ZOL (150 μg/Kg) 16	Nude/nude athymic	SH-SY-5Y neuroblastoma	5× 10^6 γδ T cells (i.v.)	From day 3 treated weekly for 4 weeks	Significant improvement of survival ($p = 0.024$; 42 vs. 48 days for untreated

[165]	hours prior to injection of γδ T cells	mice	cells (injected into adrenal gland).			and treated mice, respectively).
Parente-Pereira et al (2014) [57]	ALD or L-ALD (30 μg and 100 μg i.p and 150 μg i.v.)	SCID/Beige	SKOV-3-luc and IGROV-1-luc (injected i.p).	2 x 10^7 γδ T cells (i.p.)	Treated with ALD or L-ALD i.p., 48 and 24 hours prior to γδ T cells. Or one dose of ALD or L-ALD i.v, followed by three i.p. doses of 1 x 10^7 γδ T cells, 24, 72 and 120 hours later.	Free and liposomal ALD were shown to be equally effective at reducing tumour growth ($p < 0.001$) when delivered i.p. whereas by i.v. injection liposomal but not free ALD showed significant tumour regression ($p < 0.001$).
Hodgins et al (2016) [171]	L-ALD (0.5 μmol ALD/ mouse) i.v.	NSG	A375Pβ6 (injected i.v.)	5× 10^6 γδ T cells (i.v.)	Treated with L-ALD 24 h prior to γδ T cells. once weekly for 3 weeks	Only the group treated with both L-ALD and γδ T cells showed significant inhibition of tumour growth ($p < 0.05$).

3.8 *In vivo* toxicity of liposomal N-BPs

As mentioned above, death of mice injected with L-ZOL (0.1 μmol ZOL), without warning sign, has been reported to occur 5-7 days after injection BALB/c and outbred Sabra mice [54]. NSG mice were also found dead 5 days post i.v. injection of L-ZOL (0.1μmol ZOL) with no signs of physical abnormalities. However, NSG mice injected with mice injected with multiple doses of 0.5 μmol L-ALD showed 100% survival [171]. It has been suggested that the systemic toxicity of L-ZOL in mice is haematologically related and changes in the haematological profiles of mice injected with L-ZOL has previously been reported [54]. L-ZOL and L-ALD caused leucocytosis, neutrophilia and lymphocytopenia [171]. White blood cells count and % neutrophils increased from 0.77 ± 0.15 x 10^9/L and 66.2 ± 7.9 % in control mice to 3.22 ± 2.49 x 10^9/L ($p< 0.01$) and 92.2 ± 4.3 % ($p< 0.001$), in the L-ZOL group, with similar results observed for mice injected with L-ALD. Additionally, spleens of mice injected with L-ZOL or L-ALD weighed significantly more (0.06 ± 0.02 g) than those of control mice (0.03 ± 0.004 g) ($p < 0.01$). This is thought to de due to the trafficking of damaged macrophages containing L-ZOL or L-ALD to the spleen.

3.9 *In vivo* biodistribution of γδ T cells

In order to better understand the effect of N-BPs on γδ T cells, several studies have looked at the *in vivo* biodistribution of γδ T cells. In one study from our group, human γδ T cells were labelled with [^{111}In]tropolone, producing [^{111}In]γδ T cells [172]. Whole body SPECT/CT imaging of [^{111}In]γδ T cells, injected *via* the tail vein, in A375Pβ6 SC-tumour-bearing NSG mice, was performed to track the organ biodistribution of [^{111}In]γδ T cells over time. The mice were imaged at multiple time points up to 24 h post-injection as shown in **Figure 7**. At early time points, [^{111}In]γδ T cells were shown to accumulate in the lungs, and redistribute

overtime to the liver, spleen, and kidney. The biodistribution of [^{111}In]tropolone was examined by SPECT/CT imaging at the same time points as a control. The pattern of biodistribution for [^{111}In]tropolone was markedly different to that of the labelled γδ T cells, showing prolonged circulation and increased kidney excretion overtime. This suggests that the cells were successfully radiolabelled and the remained stable *in vivo*. Organ biodistribution of [^{111}In]γδ T cells was assessed quantitatively by gamma counting, [^{111}In]γδ T cells were cleared quickly from the blood with 6.1 – 8.8 % ID and 2.5 – 4.7 % ID present in blood at 30 min and 24 h, respectively. The liver and spleen showed high accumulation of [^{111}In]γδ T cells with an uptake of 32.7 ± 1.3 and 47.2 ± 2.6 % ID/g, respectively. Low levels of ^{111}In were present in the urine (0.11 ± 0.03) and faeces (0.43 ± 0.29 % ID). Human γδ T cells labelled with [^{111}In]oxine have also been imaged *in vivo* [142] in human patients and showed a similar pattern of biodistribution. Blood samples from these patients revealed that few cells were present in the bloodstream [142].

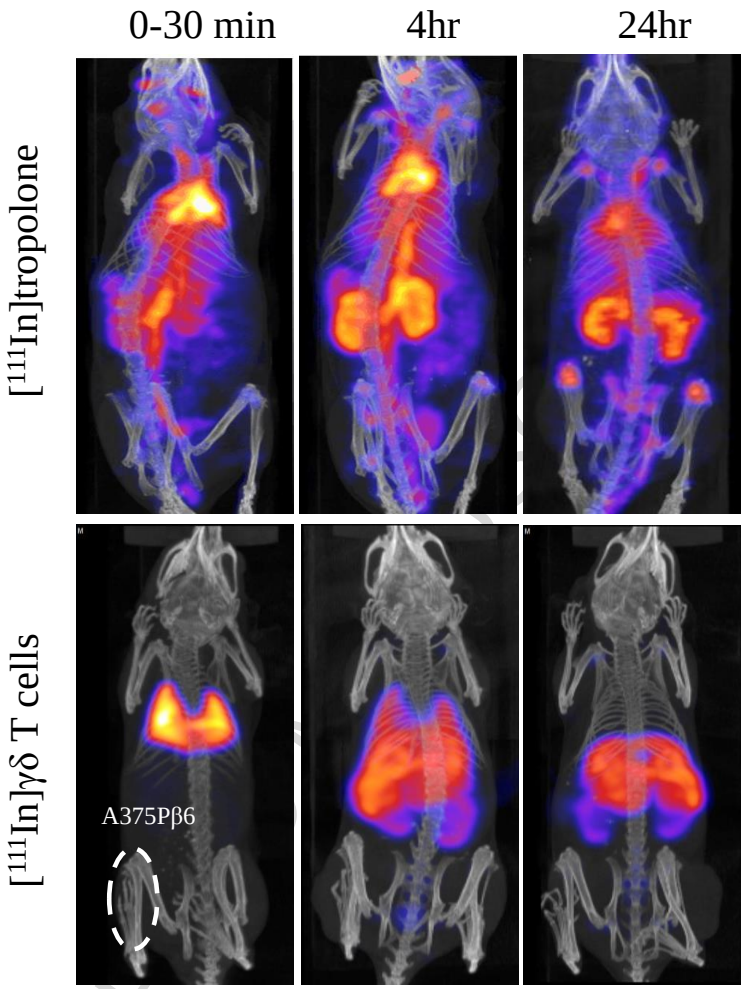

Figure 7: *In vivo* whole body 3D SPECT/CT imaging of [^{111}In]tropolone and [^{111}In]Vγ9Vδ2 T cells in subcutaneously (SC) implanted tumour-bearing SCID/Beige mice. γδ T cells were radiolabelled by incubation with [^{111}In]tropolone. Any [^{111}In]tropolone not incorporated into the γδ T cells was removed by centrifugation. Tumour bearing NSG mice were intravenously injected with 5 x 10^6 [^{111}In]γδ T cells or the equivalent amount of [^{111}In]tropolone. The mice were imaged immediately and after 4 and 24 hours. The γδ T cells go directly to the lung and then redistribute to the liver, spleen and kidneys over time. The pattern of biodistribution for [^{111}In]tropolone was markedly different to that of the [^{111}In]γδ T cells, suggesting that the cells were stably labelled and have been successfully purified from any free [^{111}In]tropolone.

L-ALD was used as a monotherapy and in combination with *ex vivo*-expanded Vγ9Vδ2 T cells in an experimental metastatic lung model with the αvβ6 positive A375Pβ6 melanoma cell line in NOD-SCID gamma (NSG) mice [173]. On day 6, all four groups had the same average tumour size (~1.3 x 10^6 photons, as determined by bioluminescence imaging). L-ALD or γδ T cells as monotherapies did not result in a significant reduction in tumour growth. Mice pre-treated with L-ALD 24 h prior to injection of γδ T cells showed a significant reduction in tumour growth, with tumour sizes of 7.53 x 10^7 ± 2.02 x 10^7 compared to 1.42 x 10^9 ± 6.38 x 10^8 photons for naïve tumours on day 27 (**Figure 8**). IFN-γ serum levels were measured on day 27. Mice pre-treated with L-ALD prior to γδ T cells had levels of 32.6 ± 19.3 and 12.3 ± 4.4 pg/ml ($p < 0.05$), respectively, compared to only 6.5 ± 0.9 pg/ml in γδ T cells-only treated group, mirroring the significant reduction in tumour growth observed in these groups.

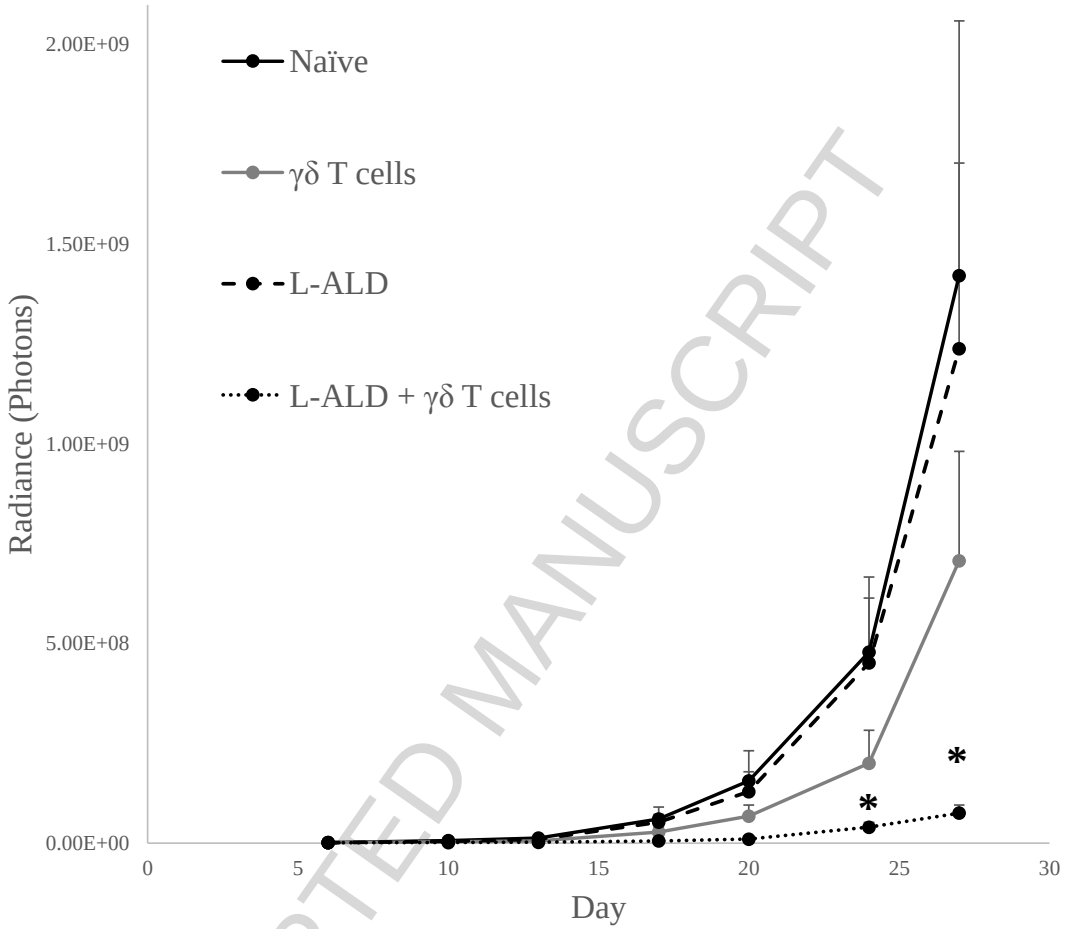

Figure 8: ***In vivo*** **tumour therapy study.** Experimental metastatic lung A375Pβ6 tumour bearing mice were treated intravenously on day 6 with L-ALD, (0.5 µmol ALD/mouse), 1 x 10^7 γδ T cells/mouse or were pre-treated with L-ALD 24 h prior to injection of γδ T cells. Three similar treatments were given intravenously at one week intervals, commencing on day 6 post-tumour inoculation. Tumour progression was monitored by bioluminescence imaging. A significant reduction in tumour growth was observed for the L-ALD/γδ combinatory immunotherapy group compared to control mice or those treated with monotherapy of γδ T cells, L-ALD. Data was expressed as mean ± SEM (n=7). *$p < 0.05$, (one-way ANOVA vs. naïve) [173].

3.10 Active targeting of liposomal N-BPs

Targeted liposomes have been formulated in order to try and increase the efficacy of the γδ T cell and L-ALD combinatory immunotherapy. The αvβ6 integrin specific peptide, A20FMDV2 was conjugated to the surface of L-ALD to produce t-L-ALD [173]. The ability of both L-ALD and t-L-ALD to sensitise cancer cell lines to destruction by Vγ9Vδ2 T cells was then tested by assessing cell viability and γδ T cell-derived IFN- γ production. The αvβ6

positive cell line A375Pβ6 was used in this assay. As shown in **Figure 9**, none of the treatments in isolation caused toxicity. However, when the cells were pre-treated with free or liposomal ALD, and were subsequently treated with γδ T cells, a significant decrease in cell viability was observed. t-L-ALD in combination with γδ T cells led to significantly lower cell viability than L-ALD at both 30 μM ($p < 0.001$) and 60 μM ($p < 0.01$) concentrations. To further confirm the increased sensitivity of αvβ6 positive cancer cells to γδ T cells when treated with t-L-ALD as compared to L-ALD, the IFN-γ release from the γδ T cells was quantified. Significantly higher amounts of IFN- γ were released when the γδ T cells were co-cultured with cells pre-treated with t-L-ALD as compared to L-ALD ($p < 0.001$ and $p < 0.001$ for 30 μM and 60 μM, respectively). This finding is in agreement with the results obtained by the MTT assay.

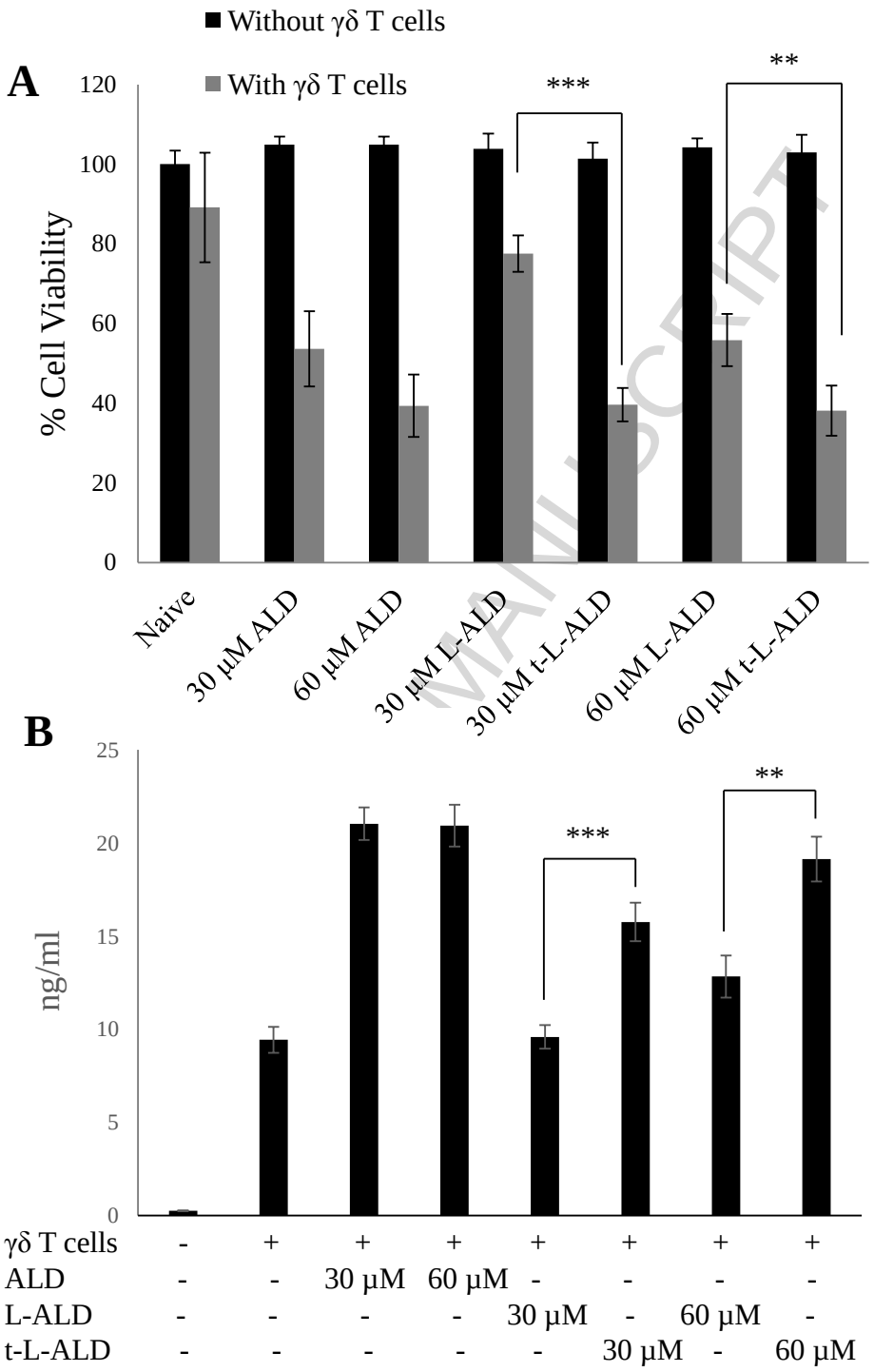

Figure 9: The ability of L-ALD and t-L-ALD to activate Vγ9Vδ2 T cells. (A) Cells were treated with ALD, L-ALD or t-L-ALD for 24 hours at 30 or 60 µM for 24 h. The treatments were then removed and replaced with 2 x 10^5 γδ T cells for a further 24 h before a MTT assay was performed. t-L-ALD increased the sensitivity of A375Pβ6 cells to γδ T cells compared to L-ALD (grey bars). ALD, L-ALD or t-L-ALD did not cause cytotoxicity alone at the concentrations used (black bars). **(B)** IFN-γ ELISA was performed on supernatant removed prior to the MTT assay. t-L-ALD led to higher release of IFN-γ from γδ T cells than L-ALD. Data was expressed as means ± SD (n=5). *$p < 0.05$, (one-way ANOVA L-ALD vs. t-L-ALD) [173].

Conclusions

N-BPs have been shown to have anti-cancer activity both as a monotherapy and in combination with γδ T cells. Due to the biodistribution of N-BPs *in vivo*, encapsulation of N-BPs in a nanoformulation is required for their use in the treatment of non-osseous tumours. Toxic side effects have been observed *in vivo* when ZOL and ALD were encapsulated into liposomes. L-ALD was shown to be better tolerated than L-ZOL. *In vivo* biodistribution and therapy studies performed by this group have shown promising results when L-ALD and γδ T cells are used in combination for the treatment of experimental metastatic lung tumours in immunocompromised mice. The use of αvβ6 ligand targeted L-ALD has shown to be more efficacious than non-targeted liposomes when used in combination with γδ T cells *in vitro*. Both murine and human studies have shown γδ T cell immunotherapy to be a well-tolerated and efficacious cancer therapy. Further work needs to be performed to understand the interaction of N-BPs with γδ T cells *in vivo* in order to fully realise their potential as a cancer treatment. While several different liposomal and non-liposomal N-BPs formulations have been used in combination with γδ T cells for different types of tumours and in different treatment protocols, optimisation of these are required to determine the most efficacious use of this combinatory immunotherapy. Additionally, prior to liposomal N-BPs being used clinically, the increased toxicity of N-BPs when encapsulated into liposomes needs to be studied further to ensure that their use in human patients is safe.

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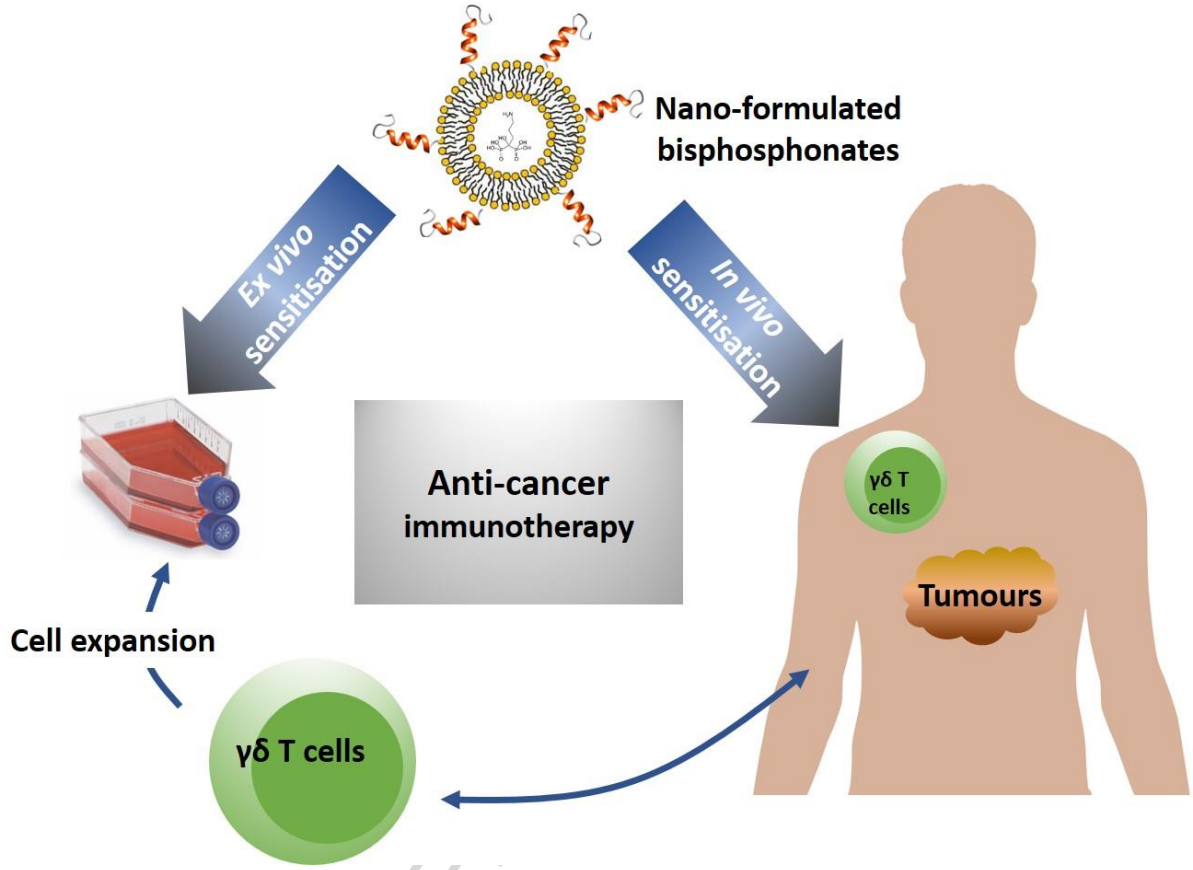

Graphical abstract